Bacteriocins from lactic acid bacteria

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CHAPTER II

General, high yield, and rapid purification method for small, hydrophobic, cationic bacteriocins.

Purification of lactococcin B from *Lactococcus lactis* subsp. *cremoris* 9B4 and pediocin PA-1 from *Pediococcus acidilactici* PAC1.0.

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SUMMARY

Lactococcin B and pediocin PA-1, two small, approximately 5 kDa hydrophobic positively charged bacteriocins, produced by *Lactococcus lactis* subsp. *cremoris* 9B4 and *Pediococcus acidilactici* PAC1.0, respectively, were purified by ethanol precipitation, preparative isoelectric focusing and ultrafiltration. The procedure leads to reproducible high final yields (41% for lactococcin B and 29% for pediocin PA-1) in comparison to the low yields (from smaller then 1% to maximally 5%) when column chromatography is used for purification. Specifically, during isoelectric focusing no loss of bacteriocin activity occurs. The method should be generally applicable to small hydrophobic cationic bacteriocins. The *N*-terminal amino acid sequence of lactococcin B was determined. The sequence of the first 21 amino acids was: Ser-Leu-Gln-Tyr-Val-Met-Ser-Ala-Gly-Pro-Tyr-Thr-Trp-Tyr-Lys-Asp-Thr-Arg-Thr-Gly-Lys, which is in agreement with a proteolytic maturation step behind the consensus glycine doublet of the leader peptide.

INTRODUCTION

The ability of lactic acid bacteria (LAB) to inhibit the growth of other bacteria has been known for many years (26). A wide variety of compounds, such as low-molecular weight antibiotics, metabolic (end-) products, enzymes, defective bacteriophages, lytic agents and bacteriocins, are responsible for this antagonistic activity. Of the criteria defined by Tagg *et al*. (31) for a bacteriocin: (i) narrow inhibitory spectrum of activity against (closely) related bacterial species; (ii) the presence of an essential, biologically active protein moiety; (iii) bactericidal mode of action; (iv) attachment to specific cell receptors; and (v) plasmid-borne genetic determinants, usually only the second and third are taken into account nowadays. There are several examples of bacteriocins with a broad inhibitory spectrum of activity including unrelated species (f.i. 24), bacteriocins that act in a receptor-independent way (9,11), and bacteriocins whose genetic determinants are located on the chromosome (17,22). Several bacteriocins of LAB have been characterized biochemically and genetically and in a number of cases their mode of action has been studied (for recent reviews see 7,16,18,19).

The ability of LAB to inhibit other bacteria by producing bacteriocins is of special interest to the food and feed industry, since some of these bacteriocins could potentially be used as food preservatives (5,25,30).

In our laboratory the operons containing genes for production of and immunity against lactococcin A, lactococcin B and lactococcin M (34,35) were cloned from *L.lactis* subsp *cremoris* 9B4 and sequenced. Only lactococcin A has been purified and an *N*-terminal sequence has been determined (15), revealing that the lactococcin A structural gene specifies a prebacteriocin which is processed behind a glycine doublet to produce the mature form.

Because bacteriocins are secreted into the growth medium, most approaches for purification start with a concentration step from the culture supernatant, such as salt precipitation (e.g. ammonium sulphate; 15,17), acid precipitation (12), or by extraction with organic solvents (33). Subsequently, several chromatographic steps (including size exclusion, adsorption, and/or hydrophobic interaction) are necessary to achieve significant purification of the bacteriocins (23).

In order to be able to study their mode of action, we developed a purification procedure that should, in

principle, be applicable to all small, hydrophobic, cationic bacteriocins and that omits column chromatography. The procedure can easily be scaled-up for large scale purification for industrial purposes. It allowed the purification of lactococcin B, a bacteriocin that proved refractive to purification using column chromatography. The purified lactococcin B and pediocin PA-1 obtained in this way have been used to study their mode of action (3,37).

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions.

The bacterial strains and plasmids used in this study are listed in Table 1. *L.lactis* was cultured in M17 broth (30), Elliker lactic broth (8), or in chemically defined medium (CDM) supplemented with 0.5 % glucose or lactose. CDM was composed of the following: buffer (21 g Na-ß-glycerophosphate, 1 g KH_2PO_4, 0.6 g $(NH_4)_3$citrate, 1g Na-acetate), amino acid mix (290 mg Tyr, 250 mg Cys, 237 mg Ala, 390 mg Gln, 350 mg Asn, 125 mg Arg, 437 mg Lys, 212 mg Ile, 125 mg Met, 275 mg Phe, 337 mg Ser, 225 mg Thr, 50 mg Trp, 325 mg Val, 175 mg Gly, 150 mg His, 475 mg Leu and 675 mg Pro), vitamin mix (4 µg pyridoxal, 2 µg nicotinic acid, thiamine dichloride, riboflavin, Ca-pantothenate, folic acid, 20 µg Na-p-aminobenzoate, biotin, 10 µg orotic acid, 2-deoxythymine, inosine, pyridoxamine dichloride and 5 µg DL-6.8-thioctic acid), metal mix (40 mg $MgCl_2.6H_2O$, 7.4 mg $CaCl_2$, 1 mg $FeCl_2.4H_2O$, 1 mg $ZnSO_4.7H_2O$, 500 µg $CoCl_2.6H_2o$, 20 µg $CuSO_4.5H_2O$ and 2.8 mg $MnSO_4.H_2O$) and base mix (2 mg adenine, uracil, xanthine and guanine) per liter, pH 6.4. Pediococci were grown in casein glucose broth (1) or in MRS broth (6) containing 0.5% glucose. 1.5% agar was added to the media for plating. *L. lactis* subsp. *lactis* IL1403(pMB580) (35) and IL1414 (pMGCT7'580, pIpS) were used as lactococcin B producers, *L. lactis* IL1403 was the indicator strain for

Table 1: Bacterial strains and plasmids.

Bacterial strain or plasmid	Relevant properties	source or reference
Bacteria		
Lactococcus lactis		
IL1403	plasmid free indicator strain for lactococcin B	4
IL1414	IL1403 derivative, containing lactose plasmid	4
Pediococcus		
acidilactici PAC 1.0	wild type pediocin PA-1 producer	24
pentosaceus PPE 1.2	indicator strain for pediocin PA-1	24
Plasmids		
pMB580	Em^r, containing the lactococcin B operon	35
pIpS	Sp^r, pIlPol-derivative, carrying T7 RNA polymerase gene	this work
pMGCT7'580	Cm^r, pMG36CT-derivative, containing the lactococcin B operon under control of the bacteriophage T7 promoter	this work
pIlPol	Em^r, containing T7 RNA polymerase gene	40
pMG36CT	Cm^r, pMG36e-derivative, lactococcal gene expression vector	C.M. Franke

Em: erythromycin; Sp: spectinomycin; Cm: chloramphenicol; r: resistance.

lactococcin B. *P. acidilactici* PAC 1.0 and *P. pentosaceus* PPE1.2 were the pediocin PA-1 producer (24) and indicator, respectively. Erythromycin (Em) was used at a final concentration of 5 µg/ml for both *L.lactis* and *Pediococcus*. Spectinomycin and chloramphenicol were used at final concentrations of 200 µg/ml and 5 µg/ml for *L.lactis*, respectively.

Bacteriocin assay.

The agar spot test was used to determine bacteriocin activity (37). In short, 16µl of a solution containing bacteriocin was spotted on an agar plate in serial two-fold dilutions in demineralized water, containing 0.5% Tween 20, and, for lactococcin B, 1 mM DTT. After the liquid had been completely adsorbed by the agar, a topagar layer seeded with 0.1% of an overnight culture of the indicator was poured over the plates. The plates were incubated for 12-14 hrs at 30 °C and examined for zones of growth inhibition. The highest dilution which gave a distinct zone of inhibition after 16 h was defined as 1 arbitrary unit (AU). Overlayers on colonies were done as follows: Colonies were treated with chloroform vapour for 15 min. After exposure of the plates to air for another 30 min., softagar seeded with 0.1% of an overnight culture of the indicator was poured on the plates. After 12-14 hrs. of incubation at 30 °C the plates were examined for zones of growth inhibition.

Determination of optimal conditions for production of lactococcin B.

An overnight culture of *L.lactis* IL1403(pMB580) was used to inoculate (to approx. 10^6 CFU/ml) several samples of GM17 broth containing increasingly more ß-glycerolphosphate (from 0-2%, in steps of 0.5%), Elliker lactic broth, CDM, a GM17 dialysate, or double M17 with 0.5% glucose (G2M17). The inoculated media were incubated at 30 °C and samples were removed to determine bacteriocin activity and cell density (measured as optical density at 660nm.).

Lactococcin B production.

1 liter G2M17 was inoculated at 1% with an overnight culture of *L.lactis* IL1403(pMB580) and grown at 30 °C until early stationary phase (approx. 5.5 hrs.). Then, cells were removed by centrifugation and the supernatant was sterilized by heating at 100 °C for 30 min. Activity did not decrease during this step. Purification was done according to the procedure below.

Pediocin PA-1 production.

For purification of pediocin PA-1, 1 liter of dialysed casein glucose broth (2) was inoculated with 50 ml overnight culture of *P. acidilactici* PAC1.0 and grown overnight at 37 °C. Cells were removed by centrifugation and the supernatant was filter sterilized (0.45 µm Schleicher and Schuell, Inc., Keene, N.H.). The activity before and after filter sterilization did not differ.

General purification method.

The bacteriocins were precipitated by mixing the supernatant with an equal volume of cold (-20 °C) 96% ethanol. After 3 hrs at -20 °C the precipitate was collected by centrifugation (10.000 rpm, 30 min.) and resuspended in 50 ml demineralized water. Ampholytes (Bio-Rad, Richmond, Calif., pH range 3-10) were added to 1%, Tween-20 (Tween-80 for pediocin PA-1) to 0.1% and glycine to 5% and the final volume was made up to 55 ml. Subsequently, the proteins were loaded in the Rotofor isoelectric focusing cell (BioRad) for preparative isoelectric focusing at a constant power of 12W for approx. 5.5 hrs (until a plateau of constant voltage was obtained). The fractions were collected and the pH of each fraction was adjusted to approximately 7. The activity in each fraction was tested by the agar spot test. Fractions containing bacteriocin were mixed and ultrafiltrated through a membrane with a 3-kDa molecular weight cut off in a Spin-x UF concentrator (Corning Costar Corporation, Cambridge, MA) to remove ampholytes. Purity of the samples was tested by tricine-SDS-PAGE (29) and subsequent silver staining of the gel (BioRad). Protein concentrations were determined spectrophotometrically at 280nm.

Overexpression of lactococcin B using bacteriophage T7 RNA polymerase expression system.

Overexpression of lactococcin B was established by using the T7 overexpression system for *L.lactis* essentially as developed by Wells *et al.* (40). The system was adapted as follows:

In plasmid pILpol, containing the T7 RNA polymerase gene under control of the lactose-inducible lactococcal *lac* promoter, the the MLS marker was

replaced by a *Cla*I-*Nde*I fragment from pDL55 containing the spectinomycin resistance marker AAD(9) (20), resulting in plasmid pIpS. Instead of pMG280 (40) as the carrier of the *lac* operon, strain IL1414 (4; a derivative of IL1403 containing a lactose plasmid) was used. The T7 expression vector, containing *lcnB* and *lciB* under control of the T7 promoter, was constructed by cloning the *Hin*dIII-*Xba*I fragment from pT7'580 (35) in pMG36CT cut with the same restriction endonucleases, resulting in pMGCT7'580. IL1414 was transformed with pIpS and pMGCT7'580. The resulting transformant was grown

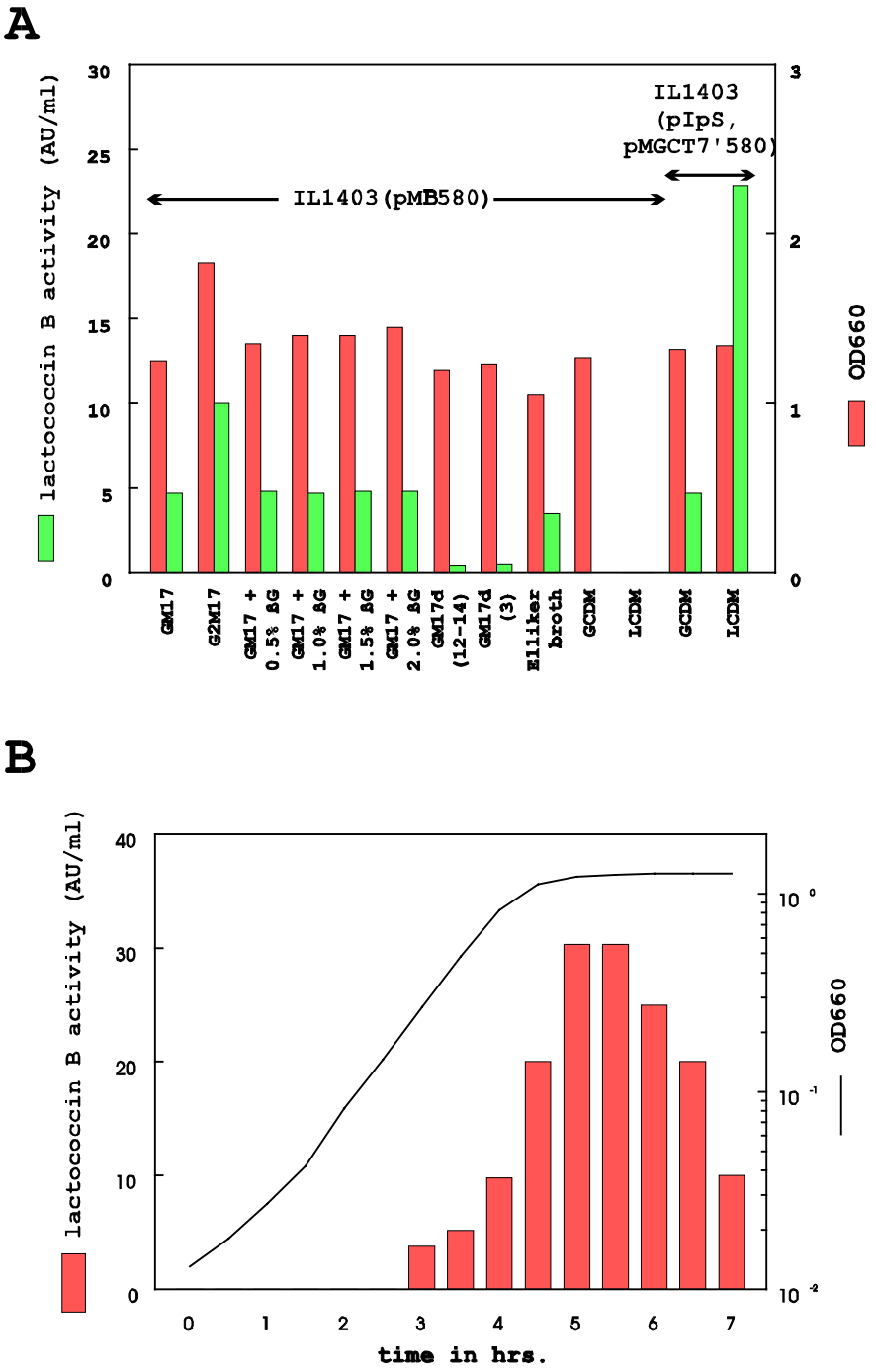

Figure 1: A: Lactococcin B production in various media. A 1% inoculum of *L.lactis* IL1403 (pMB580) was grown at 30 °C until early stationary phase in the media indicated. At that time samples were removed and the amount of bacteriocin was determined as well as the OD660. B: Lactococcin B production during growth of a 1% inoculum of *L.lactis* IL1403 (pMB580) in G2M17 at 30 °C. With 30 min. intervals the optical density at 660 nm. was determined as well as the amount of bacteriocin by the agar spot test.

on medium containing lactose, spectinomycin and chloramphenicol. Transformation of *L.lactis* was done as described earlier (21). General DNA cloning and manipulation techniques were carried out essentially as described by Sambrook *et al.* (28).

Purification of overexpressed lactococcin B.

Culture supernatants of IL1414(pIpS, pMGCT7'580) grown to early stationary phase on CDM containig 0.5% lactose (LCDM) were freeze dried (Lyph-lock ®4.5, Labconco, Kansas City, MI). After redissolving the freeze dried material in one-fiftieth of the original volume, the sample was ultrafiltrated as described above.

***N*-terminal amino acid sequencing.**

N-terminal amino acid sequencing was carried out by means of Edman degradation on an automated sequenator (Model 477A, Applied Biosystems, Foster City, Cal.) using protocols, chemicals and materials from Applied Biosystems. The fenylthiohydantoin-derived amino acids were identified using on on-line coupled HPLC (Model 120A ABI, Applied Biosystems).

RESULTS

Optimal conditions for production of lactococccin B.

Growth of *L.lactis* IL1403(pMB580) and production of lactococcin B were studied in various media. Figure 1A shows that production was highest in G2M17. The bacteriocin was produced during logarithmic growth and highest activity was found in the early stationary phase (Fig. 1B). After that activity declined, probably by readsorption of lactococcin B to the producer cells, or by inactivation of the bacteriocin by the proteolytic/ peptidolytic system of the producer. Contrary to what was reported by Geis *et al.* (10), production in Elliker lactic broth was lower than on GM17, even with the original strain *L.lactis* subsp. *cremoris* 9B4. No production was observed on CDM containing glucose (Fig. 1A). Because proteins and peptides present in a rich medium like GM17 complicate purification of bacteriocins, production was also tested in a GM17 dialysate. A 10 times concentrated M17 solution was dialysed in a dialysis bag with a molecular weight cut off of 12 to 14 kDa [resulting in GM17d(12-14)] or of 3 kDa [resulting in GM17d(3)]. To the dialysate

Table 2: Purification of lactococcin B and pediocin PA-1.

sample/fraction	volume (ml)	OD_{280}	total activity (AU)[a]	specific activity (AU/ml*OD_{280})	purification (fold)	yield (%)
Pediocin PA-1						
culture supernatant	1000	3.3	$1.6 * 10^6$	$4.8 * 10^2$	1	100
ethanol precipitate	40	0.7	$5.1 * 10^5$	$1.8 * 10^4$	37	32
rotofor samples	11.4	0.7	$4.7 * 10^5$	$6.3 * 10^4$	131	29
retentate	11.4	0.5	$4.6 * 10^5$	$8.1 * 10^4$	169	29
Lactococcin B						
culture supernatant	1000	35	$1.0 * 10^4$	$0.3 * 10^0$	1	100
ethanol precipitate	50	50	$3.0 * 10^4$	$1.2 * 10^1$	40	300
rotofor samples	20	3.1	$2.9 * 10^4$	$4.8 * 10^2$	1600	299
retentate	2.5	2.7	$4.2 * 10^3$	$6.1 * 10^2$	2033	41
Lactococcin B/CDM						
culture supernatant	1000	6.0	$5.0 * 10^4$	$8.3 * 10^1$	1	100
lyophilisate	50	5.5	$7.3 * 10^4$	$6.6 * 10^2$	8	146
retentate	2.5	2.5	$6.9 * 10^4$	$5.6 * 10^4$	675	138

[a] total activity in arbitrary units (AU) was determined in an agar spot test using *P.pentosaceus* PPE1.2 (for pediocin PA-1) or *L.lactis* IL1403 (for lactococcin B) as the indicator strain.

glucose was added to 0.5% and the lactococcin B producer was inoculated at 1%. Although both medium dialysates supported good growth of the producing strain, only 10% of the bacteriocin activity on GM17 was obtained (Fig. 1A). For purification of lactococcin B, therefore, G2M17 was taken.

Purification of pediocin PA-1.

After overnight growth of *P. acidilactici* PAC 1.0 the supernatant contained $1.6*10^6$ AU of activity (Table IIa). About 32% could be precipitated with cold ethanol (Table IIa). The precipitate was dissolved in demineralized water and, after addition of carrier ampholytes, Tween 80, and glycine, subjected to preparative isoelectric focusing. After 5.5 hrs of running at a constant power of 12W, separation was complete (as indicated by the voltage reaching a plateau). Spot testing indicated that activity was present in fractions 17-20 with highest activity in fraction 20 (Fig. 2A). The pI of pediocin PA-1 was estimated to be approximately 9. The active fractions were pooled and ultrafiltrated. During isoelectric focusing almost no activity was lost and after ultrafiltration all activity remained. After these three steps the bacteriocin was pure as judged by tricine-SDS-PAGE and silver staining (Fig. 3, lane 1).

Purification of lactococcin B.

The supernatant after 5.5 hrs of growth of a 1% inoculum of *L.lactis* IL1403(pMB580) in G2M17 contained $1.0*10^4$ AU/l of activity on *L.lactis* IL1403 as the indicator (see Table IIb). However, after ethanol precipitation $3.0*10^4$ AU/l were present in the precipitate. This increase in activity is indicative of multimer formation, which has been reported for other bacteriocins (23).

After 5.5 hrs of isoelectric focusing, the activity was present in fractions 14-20, being highest in fraction 18 (Fig. 2B). The pI of the bacteriocin was estimated to be about 8.5. Fractions 14-20 were pooled and ultrafiltrated. The bacteriocin solution was devoid of ampholytes as shown by tricine-SDS-PAGE and subsequent silver staining (Fig 3, lane 2). During isoelectric focusing hardly any loss of activity was observed but, in contrast to pediocin PA-1, losses were considerable after ultrafiltration (Table IIb). The amount of lactococcin B purified was insufficient to determine an *N*-terminal amino acid sequence. Since the initial amount of activity was already low in supernatants of IL1403(pMB580), lactococcin B was overexpressed using the bacteriophage T7 RNA polymerase expression system.

Overexpression and purification of lactococcin B on chemically defined medium.

Apart from the strong T7 promoter, plasmid pMGCT7'580 also contains the normal lactococcin B promoter. Therefore, lactococcin B production on GM17 and LM17 could be compared. On GM17 plates lactococcin B production by IL1414(pIpS, pMGCT7'580) was similar to that of IL1403(pMB580) as judged by the size of the zone of growth inhibition (halo) around a producing colony. However, on LM17 production of lactococcin B by IL1414(pIpS, pMGCT7'580) was similar to the amount produced on GM17 by the same strain: no overexpression was obtained. As documented above, production under control of the lactococcin B promoter was only observed in rich media. Also, no production was observed in GCDM by IL1414(pIpS, pMGCT7'580). However, the strain grown in CDM containing lactose produced amounts of lactococcin B similar to that in LM17. Since CDM does not contain any constituents with a molecular mass higher than approximately 1.2 kDa, and lactococcin B has a mass of approximately 5 kDa, the peptide was purified from the supernatant of IL1414(pIpS, pMGCT7'580) grown in LCDM by the method described here. The supernatant was freeze dried, and ultrafiltrated after redissolving in water. The purified sample of lactococcin B (yield 95%, Table IIc) was subjected to tricine-SDS-PAGE and silver staining and shown to be pure (Fig. 3, lane 3).

Purified lactococcin B was subjected to *N*-terminal amino acid sequencing. The first 21 *N*-terminal amino acids were: Ser-Leu-Gln-Tyr-Val-Met-Ser-Ala-Gly-Pro-Tyr-Thr-Trp-Tyr-Lys-Asp-Thr-Arg-Thr-Gly-Lys. Comparing this sequence with the sequence deduced from the *lcnB* gene revealed that prelactococcin B, like many other bacteriocins (13), is processed behind a glycine doublet.

DISCUSSION

Lactococcin B production is medium dependent. Highest production is obtained in rich media like M17. Bacteriocin production is even higher when a medium containing the double amount of M17 is used. Production is low in an M17 dialysate, indicating that a factor(s) larger than 14 kDa is required for optimal

bacteriocin production. Contrary to the results obtained by Geis *et al.* (10) production on Elliker broth was lower than on M17. An explanation might be that the constituents of the media used in these two studies came from different suppliers. Medium-dependent bacteriocin production has been observed before (f.i. 27).

L.lactis subsp. *cremoris* 9B4 produces at least three different bacteriocins: lactococcin A , B and M (34,35). All belong to the class II non-lanthionine-containing group of bacteriocins. Of these lactococcins, the mode of action of lactococcin A has been studied earlier

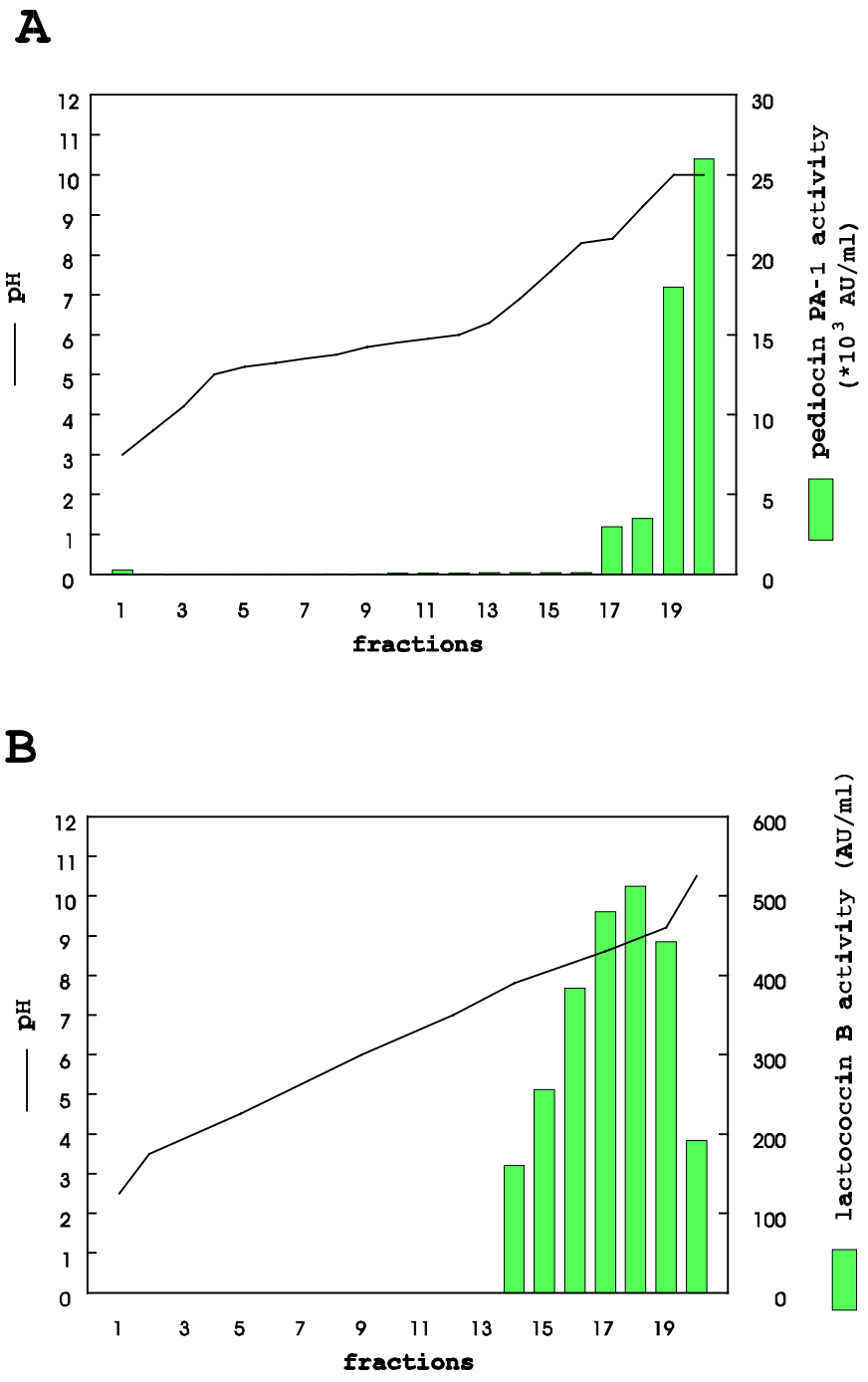

Figure 2: Activity distribution of pediocin PA-1 (A) and lactococcin B (B) after preparative isoelectric focusing. The amount (AU/ml) of bacteriocin in every fraction was determined in a spot test.

Figure 3: Silver staining of a 16% Tricine-SDS-PAA gel containing purified samples of pediocin PA-1 (lane 1), and lactococcin B (lanes 2 and 3, purified from G2M17 and CDM, respectively). lane 4: molecular weight marker (BioRad) the sizes of which are indicated in the right margin.

(36). Purified lactococcin B obtained with the purification protocol reported here has been used in mode of action studies as well (37). Both lactococcins permeabilize the cytoplasmic membrane of sensitive cells, most probably by the formation of pores. The mode of action of the third bacteriocin, lactococcin M, has not yet been determined. Two peptides are required for activity of this bacteriocin (34,39). Also, pediocin PA-1 purified with this method has been examined for its mode of action (3).

Since the use of columns to purify lactococcin B resulted in complete loss of activity, we decided to develop an alternative protocol for purification of this bacteriocin. Pediocin PA-1 has been purified previously (14), employing column chromatography. The yield in the procedure used by Henderson *et al*. was only 0.6%. With our method we repeatedly obtained very pure bacteriocin preparations with yields between 30 and 40 percent for lactococcin B and pediocin PA-1 (excluding the yield obtained using the lactococcin B overexpression system and CDM, which was 138%). Usually yields are between 0.5 and 1 percent with other purification protocols. The great advantage of the present procedure is the omission of columns. Usually, the hydrophobic bacteriocins stick to the matrix of columns causing great losses (23). Instead, we used preparative isoelectric focusing for purification with minimal losses (less than 1% for lactococcin B, 3% for pediocin PA-1, compared to 40% to 80% loss on cation and/or anion exchange columns). The increase in activity after ethanol precipitation of lactococcin B from culture supernatants is probably due to dissociation of aggregates. Almost all bacteriocins have a tendancy to aggregate (23).

We believe that the procedure described here could also be used for other (small) hydrophobic, positively charged bacteriocins. A trial with lactococcin A (previously purified by Holo *et al.* (15) with a yield of 16%) showed that it could be purified with a yield over 30% (not shown). Also, mutant lactococcin B molecules have been purified using this method (38).

Bacteriocins are of potential interest to the food industry because of their ability to inhibit food-related pathogens and spoilage organism. Some applications (e.g. mode of action studies) would require highly purified bacteriocin preparations, whereas in other applications (e.g., use as a biopreservative) it may be preferable to use a less purified sample. In both cases a high final yield is desirable. Cost and reproducibility will also influence the industries choice for a specific purification protocol. The method described here reproducibly provides high yields of purified bacteriocin and could be easily scaled-up for industrial purposes.

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